

Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons

Leila Tarsa and Yukiko Goda*

Division of Biology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0366

Edited by Charles F. Stevens, The Salk Institute for Biological Studies, La Jolla, CA, and approved November 20, 2001 (received for review October 29, 2001)

Synaptophysin is an abundant synaptic vesicle protein without a definite synaptic function. Here, we examined a role for synaptophysin in synapse formation in mixed genotype micro-island cultures of wild-type and synaptophysin-mutant hippocampal neurons. We show that synaptophysin-mutant synapses are poor donors of presynaptic terminals in the presence of competing wild-type inputs. In homogenotypic cultures, however, mutant neurons display no apparent deficits in synapse formation compared with wild-type neurons. The reduced extent of synaptophysin-mutant synapse formation relative to wild-type synapses in mixed genotype cultures is attenuated by blockers of synaptic transmission. Our findings indicate that synaptophysin plays a previously unsuspected role in regulating activity-dependent synapse formation.

culture | activity | synaptic competition

The mechanism underlying the formation of functional synaptic circuits is one of the central problems of neurobiology that remains to be resolved. In the prevailing view, growing axons are guided to their targets and undergo synaptogenesis by predominantly activity-independent processes. Subsequently, the initial rough connections are refined by activity-dependent synapse remodeling, in which active synapses are preferentially stabilized at the expense of less active synapses to generate an optimal synaptic circuit supporting nervous system function (1–3). The molecular mechanisms of synaptogenesis and synapse remodeling are best understood at the neuromuscular junction, where some of the key molecular players have been identified (4). In central neurons, however, the mechanisms that drive the formation of functional synaptic circuits remain largely unknown.

Synaptophysin I (syp) is a synaptic vesicle membrane protein that is ubiquitously expressed throughout the brain (5, 6). Despite its abundance, analysis of syp interactions with other synaptic vesicle proteins and presynaptic molecules has not revealed a clear function. A possible role for syp in regulating synaptic vesicle cycling has been suggested by the findings that antibodies to syp reduce neurotransmitter release in *Xenopus* neuromuscular synapses (7), and that peptides which interfere with syp binding to dynamin, a component of endocytic machinery, block endocytosis at squid giant synapse (8). Mice carrying a targeted deletion of the syp gene, however, do not display any obvious phenotype to support these proposals (9). The anatomical structure and protein composition of the brain seem normal, and the properties of baseline synaptic transmission and short- and long-term synaptic plasticity also are unchanged compared with wild-type mice. These results have suggested that syp function is either redundant or compensated for by other proteins (9). It also is possible that syp plays a subtle, nonessential regulatory role in some aspect of synapse function that is not apparent when comparing the differences between wild-type and mutant animals.

Here, we investigate a role of syp in synapse formation in the presence of competing wild-type inputs, a condition that has not been tested previously. We find that the extent of synapse formation is considerably reduced for syp-mutant synapses. In

homogenotypic syp-mutant cultures, however, synapse formation is similar to that observed for wild-type cultures. Interestingly, the decrease in syp-mutant synapse formation is prevented when heterogenotypic cultures are grown in the presence of tetrodotoxin (TTX) or postsynaptic receptor blockers. Our results demonstrate a role for syp in activity-dependent competitive synapse formation.

Materials and Methods

Hippocampal Cultures. Primary cultures of dissociated hippocampal neurons were prepared from late embryonic (E18–19) or newborn (P1) wild-type and syp-mutant mice as described (10). Briefly, dissected hippocampi were incubated for 30 min in 20 units/ml papain (Worthington) in Hanks' balanced salt solution containing 10 mM Hepes (pH 7.4), 0.33 mM EDTA, and 2 nM DNase. Subsequently, the tissue was triturated, and wild-type and syp-mutant cell suspensions were incubated in 3,3'-diiodadecyloxycarbocyanine perchlorate (DiO) and 1, 1'-diiodadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), respectively (described below). The dye-labeled cells were plated onto coverslips with islands of preplated glial cells. Culture medium consisted of Basal Media Eagle (Life Technologies, Rockville, MD) supplemented with 1 mM sodium pyruvate/10 mM Hepes-NaOH, pH 7.35/0.3% glucose/50 mg/ml penicillin/50 units/ml streptomycin/10% FBS. Control cultures were fed every 3 days by replacing 100 μ l of old medium with fresh culture medium. In some experiments, 1 μ M TTX was added to the culture medium on day 0 and was replenished every 4 days. In other sets of experiments, 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μ M 2-amino-5-phosphonopentanoic acid (APV) and 50 μ M picrotoxin (PCTX) were added every other day to the growth medium, starting at day 0. Cells were used after 11–14 days in culture. Data were obtained from a total of 25 culture preparations.

Astrocytes were prepared by spraying 1.2-cm diameter glass coverslips with substrate solution of rat-tail collagen (0.5 mg/ml) and poly-D-lysine (5 μ g/ml); glial cells were plated at 4,000 cells per cm^2 . Cytosine β -D-arabinofuranoside (4 μ M) was added to the culture medium after 3–4 days to prevent the overgrowth of astrocytes.

Di-I/Di-O Staining. Cultured neurons were stained with DiI or DiO according to Potter *et al.* (11). The stock solution was prepared by adding each dye to a 2.5% (wt/vol) solution of Pluronic F127 (Sigma) in dimethylformamide to a final concentration of 40 mg/ml; the solution was stored at -20°C . Before dissection, the stocks were sonicated for 15 min at 37°C . The dyes were diluted in 2 ml of culture medium to a final concentration

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: syp, synaptophysin I; TTX, tetrodotoxin; DiO, 3,3'-diiodadecyloxycarbocyanine perchlorate; DiI, 1,1'-diiodadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; syt, synaptotagmin I.

*To whom reprint requests should be addressed. E-mail: ygoda@biomail.ucsd.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

of 40 $\mu\text{g}/\text{ml}$, and the solution was filter-sterilized. Cell suspensions were incubated for 20 min at 37°C in DiO and DiI solution for wild-type and mutant cells, respectively. Subsequently, excess dye and large debris were removed by centrifuging the cell suspension through a 200 μl cushion of 7.5% BSA at $350 \times g$ for 1.5 min. The cell pellet was resuspended in culture medium. Equal numbers of labeled wild-type and syp-mutant cells were mixed and plated onto preplated glial cells at the following densities (total number of cells per well): 4,000, 6,000, 8,000, 10,000, 12,000.

Immunocytochemistry. Coverslips containing two-cell islands of wild-type and syp-mutant neurons were identified by DiI and DiO labels. The fluorescence signals were visualized on an Olympus BX50WI microscope with a $40\times$ 0.8 N.A. water-immersion objective, and images were captured with a Princeton Instruments RS-cooled charge-coupled device camera. Phase contrast ($10\times$) and differential interference contrast (DIC; $40\times$) images also were acquired to facilitate relocating the specific island on the coverslip after the immunofluorescence procedure. Samples were processed for immunohistochemistry by using standard methods. Briefly, coverslips were fixed in 4% (wt/vol) paraformaldehyde in PBS for 15 min. After permeabilization, samples were incubated in primary antibodies at 1:50 dilution for 1 h. The polyclonal syp antibody was a generous gift from T. Südhof (University of Texas, Dallas, TX), and the monoclonal synaptotagmin (syt) antibody was generated in the Goda lab. The secondary antibodies, FITC goat anti-mouse (Roche Molecular Biochemicals), and rhodamine goat anti-rabbit (Roche Molecular Biochemicals) were applied for 1 h, and the coverslips were mounted in Mowiol (Calbiochem) with p-phenylene diamine. All procedures were carried out at room temperature. The same cell pair was found on the coverslip after the immunostaining, and the fluorescence signals were acquired with the $10\times$ and $40\times$ objectives.

Data Analysis. The fluorescence images were normalized to maximal contrast, overlaid, and analyzed with the METAMORPH imaging software (Universal Imaging, Media, PA). Wild-type synapses were identified based on the colocalization of syp and syt immunofluorescence puncta, and syp-mutant synapses were identified as syt-positive puncta lacking the syp fluorescence. Out of focus regions were excluded from the analysis. To distinguish autapses from heterosynapses, only neurites with known origins were analyzed. To count the number of synapses, the field of view was divided into squares of the same area. The length of the neurites within the squares and the number of synapses along the neurites were determined. Synaptic density is shown as the number of synapses per μm of length of mutant or wild-type dendrite. The distance from the cell body to the center of the square also was documented to demonstrate that the synapse density is not different at various distances from the soma (data not shown). Results are presented as the means \pm SEM.

Results

The Basic Assay. Our assay is based on a simple synaptic circuit formed by culturing hippocampal neurons at low densities on glial micro-islands (12, 13). When two neurons settle on an island, each cell forms synapses onto itself (autapses) and onto the other cell (heterosynapses; 14). In islands containing a wild-type and a syp-mutant neuron, each cell receives both autapses and heterosynapses, which provide homogenotypic and heterogenotypic inputs, respectively (see Fig. 3*a*). Thus, it is possible to compare the extent of mutant and wild-type synapse formation under symmetrical conditions. To identify glial islands containing a wild-type and a syp-mutant neuron, dissociated hippocampal neurons were labeled with fluorescent carbocya-

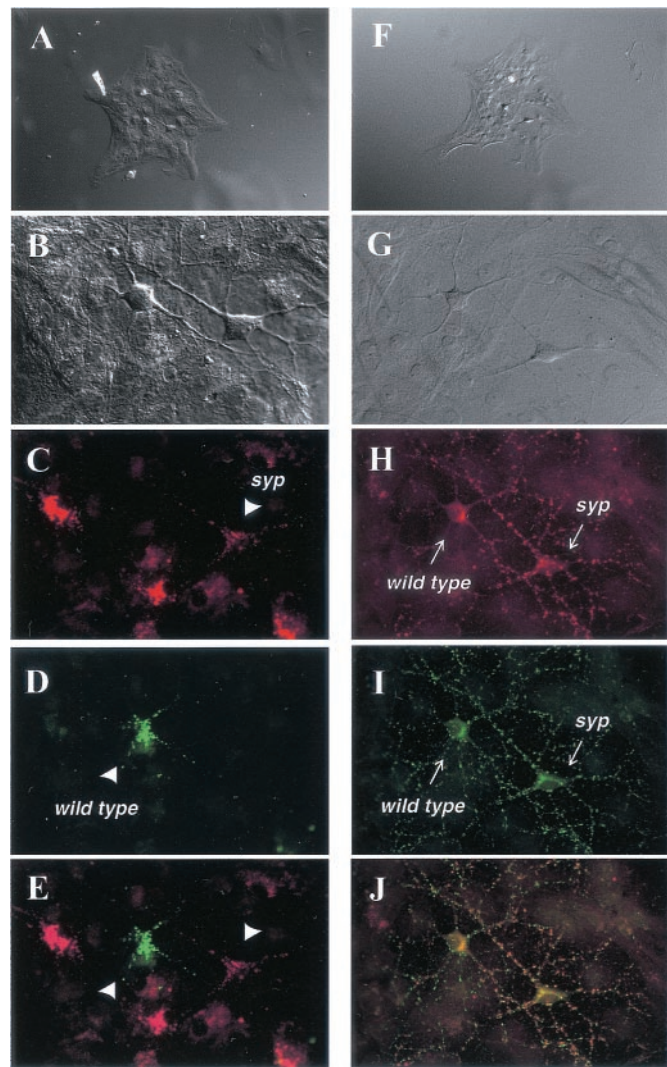


Fig. 1. Identification of wild-type and syp-mutant cell pairs. Differential interference contrast microscopy image of an island containing two neurons after 12 days in culture viewed through a $10\times$ (A) and a $40\times$ (B) objective. DiI-labeling (red) specifies the syp-mutant neuron (C, arrowhead) and DiO-labeling (green) indicates the wild-type neuron (D, arrowhead); overlaid image is shown in (E). Glial cells also take up the dye, which is noticeable especially for the DiO-labeling. After fixation and processing for immunofluorescence labeling, the same island ($10\times$ in F and $40\times$ in G) is identified by referencing the Nomarski images acquired before cell fixation. Immunofluorescence labeling for syp in rhodamine channel (H), syt in FITC channel (I), and overlay of both images (J) are shown. The arrows (I and J) indicate the syp-mutant and the wild-type cell bodies identified in C and D. Note that it is not practical to identify the two genotypes based on the lack of syp labeling because the wild-type cell receives autaptic wild-type synapses in addition to mutant synapses.

nine dyes and plated onto islands of astrocytes. After 11–14 days in culture, we identified glial islands containing a DiI-labeled wild-type and a DiO-labeled mutant neuron (Fig. 1). Although most of the dye was internalized, the genotype of the neurons was readily identified by the strong fluorescence puncta that accumulated in the soma. The distribution of wild-type and mutant synapses was examined by double immunofluorescence labeling for synaptic vesicle proteins that reliably indicate the location of synapses. The antibody against syp labeled wild-type synapses only, and anti-syt antibody labeled both wild-type and mutant synapses. DiI and DiO labeling did not interfere with immuno-

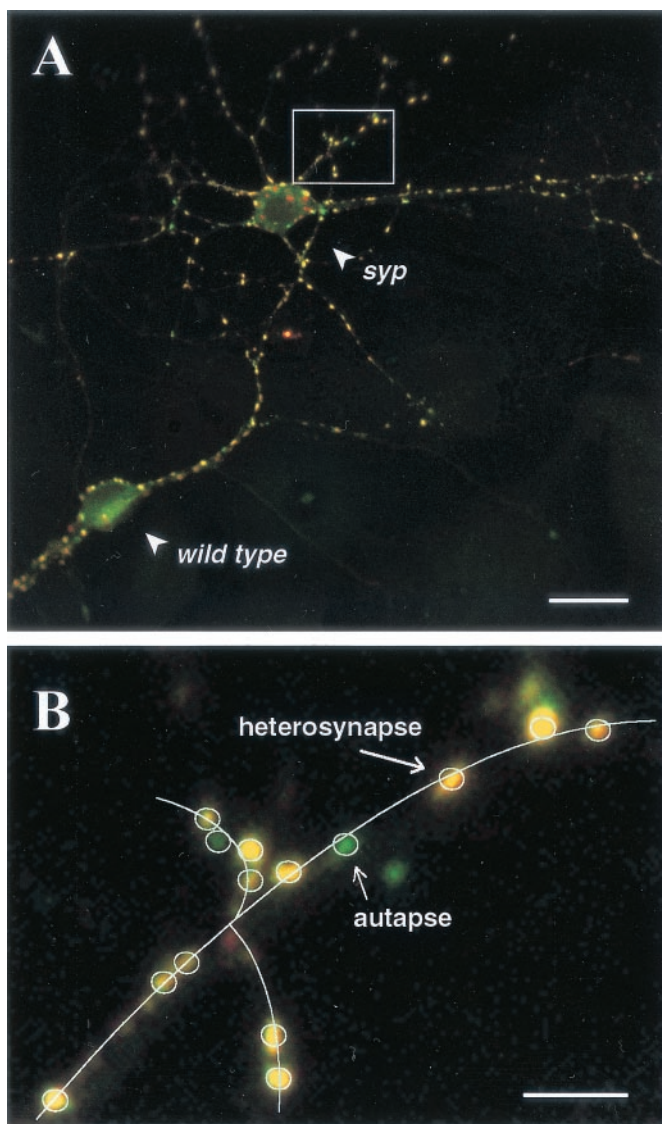


Fig. 2. Counting synapses along the syp-mutant dendrite based on overlaid images of syp and syt immunofluorescence. For the 12-day-old heterozygous cell pair shown (A), determination of autapses and heterosynapses along a mutant dendrite is illustrated for the boxed area (B). Autapses are devoid of syp fluorescence and display syt immunofluorescence (green), whereas heterosynapses are positive for both syp and syt immunofluorescence (yellow). Lines were drawn along the dendrites to determine their lengths. [Bar = 20 μ m (A) and 5 μ m (B)]. Note that several fluorescence puncta that appear after immunolabeling for syp in the rhodamine channel (red) do not contain syt. They represent less than 3% of total syp- or syt-positive fluorescence puncta (unpublished data) and have been excluded from analysis.

fluorescence localization of synaptic vesicle markers because the dyes did not persist through cell fixation and permeabilization steps. Fig. 2 illustrates how synapses that formed onto a given neuron were designated as an autapse or a heterosynapse. The example in Fig. 2B illustrates a syp-mutant dendrite. A synapse that is positive for both syt and syp will originate from the wild-type cell; therefore, such a synapse on a mutant dendrite is a heterosynapse. A synapse on a mutant dendrite that is positive for only syt originates from the mutant cell, and hence it is a mutant autapse. We refer to a “wild-type heterosynapse” as a heterosynapse formed by the wild-type neuron onto a mutant dendrite; a “mutant heterosynapse” denotes a syp-mutant synapse formed onto a wild-type neuron (Fig. 3A). The designation

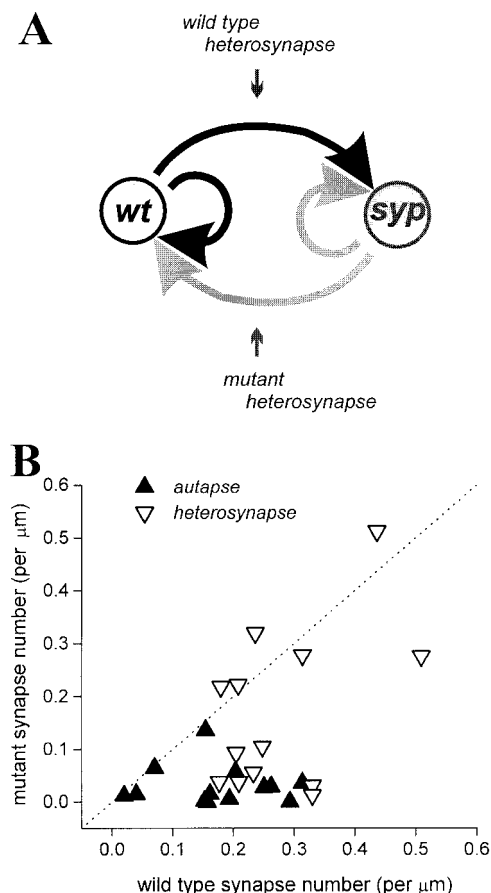


Fig. 3. Syp-mutant synapses form at lower densities relative to wild-type synapses in a two-cell circuit. (A) Schematic of the two-cell circuit illustrating the symmetry of autaptic and heterosynaptic inputs between the wild-type and the mutant cell. (B) Comparison of wild-type vs. mutant synapse densities (number of synapses per μ m of dendritic length) for 13 cell pairs from a total of 1,410 synapses (424 autapses and 986 heterosynapses). Each point represents either autapse (\blacktriangle) or heterosynapse (∇) density for a heterozygous cell pair. Most points fall on or below the line with a slope of 1 (dotted line); therefore, wild-type synapses are favored over syp-mutant synapses for the majority of cell pairs.

of wild-type and mutant synapses depends on the degree of colocalization of syp and syt immunofluorescence puncta. In our experimental conditions, $\approx 97\%$ of fluorescence puncta labeled for both syt and syp in wild-type cultures (data not shown). In addition, we confirmed that syp-mutant cultures do not display syp immunofluorescence puncta (data not shown).

The Extent of Synapse Formation Is Impaired for syp-Mutant Neurons.

To compare the extent of wild-type vs. syp-mutant synapse formation, we determined the mean numbers of autapses and heterosynapses formed per unit of dendritic length (see *Methods*). Fig. 3B summarizes the wild-type vs. mutant relation for symmetrical autapses and heterosynapses formed between wild-type and mutant cell pairs ($n = 13$) after 11–14 days in culture. Although some cell pairs displayed balanced wild-type and mutant synapse formation for both autapses and heterosynapses, overall, wild-type synapses formed at significantly higher densities compared with mutant synapses. The mean wild-type and mutant autapse densities were 0.17 ± 0.03 and 0.03 ± 0.01 (number of synapses per μ m of dendritic length; $P < 0.05$), respectively. The mean wild-type and mutant heterosynapse densities were 0.28 ± 0.03 and 0.17 ± 0.04 (per μ m dendrite; $P <$

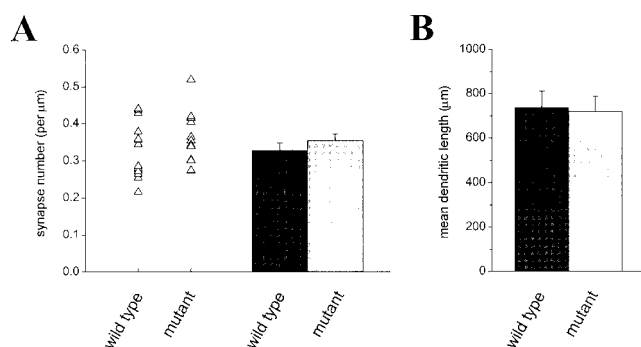


Fig. 4. Syp-mutant neurons display normal synapse formation in homogenotypic cultures. (A) Mean synapse density (number of synapses per μm of dendritic length) of wild-type or mutant autapses formed in single-cell islands of homogenotypic cultures are shown as a scatter plot (Left) or a bar graph (Right). No significant difference was observed between the two groups of cultures grown in parallel ($n = 14$ cells from each genotype; $P > 0.3$). Data are from total of 1,254 wild-type synapses and 1,176 mutant synapses. (B) Mean dendritic lengths, as determined by mitogen-activated protein 2 immunofluorescence, are not significantly different between wild-type and mutant autapses ($n = 24$ from each genotype; $P > 0.5$).

0.05), respectively. These results indicate that syp-mutant cells have a reduced capacity for synapse formation compared with the wild-type neurons in heterogenotypic cell pairs.

We used the mean synapse density per unit of dendritic length as a measure of synapse formation because it was not possible to count the total number of each synapse type for every cell pair analyzed. Although we attempted to score synapses along most of the dendrites discernible for a given neuron, some dendritic regions were not acceptable for analysis. These regions included those of high-fluorescence intensity on dendrites proximal to the cell body and dendrites above densely packed glial cells. To ensure that occasional exclusion of some dendritic lengths did not introduce a significant bias in our analysis, synapse density along a dendrite was measured as a function of the radial distance from the cell body. There were no significant differences in synapse densities measured at various locations for both wild-type and mutant dendrites (data not shown).

Synapse Formation in Homogenotypic Culture Is Unimpaired in syp Neuron. A previous study has reported that neuronal connectivity is apparently normal in the brains of syp-mutant mice (9). Our observed reduction in syp-mutant synapses may result from a unique configuration of heterogenotypic cell pairs where each cell is innervated by both wild-type and mutant synapses. Thus, we compared the synapse density of autapses in single-cell islands of homogenotypic wild-type and syp-mutant cultures grown in parallel. Fig. 4A illustrates that autapse density was not significantly different between the wild-type (0.33 ± 0.02 synapses per μm of dendrite, $n = 14$) and mutant cells (0.35 ± 0.02 synapses per μm of dendrite, $n = 14$; $P > 0.3$) after 13 days in culture. Although we did not detect a change in the synapse density along the dendrite, it remains possible that the mutant cell displays a reduction in the overall extent of synapse formation if the dendrite elongation was impaired. Total dendritic length, therefore, was measured by tracing the MAP2 immunofluorescence. No significant differences were observed between wild-type and syp-mutant neurons, which were $737 \pm 73 \mu\text{m}$ and $719 \pm 69 \mu\text{m}$, respectively (Fig. 4B; $n = 24$ for each genotype; $P > 0.5$). Synapse formation in syp-mutant cells, therefore, is unaltered in homogenotypic cultures, consistent with a previous study in which syp knockout mice displayed no obvious phenotype (9).

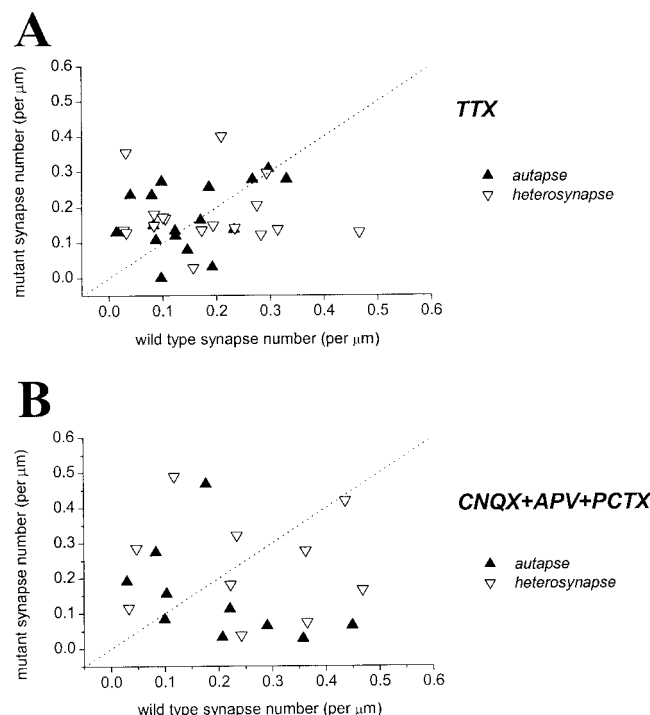


Fig. 5. The bias toward wild-type synapses in heterogenotypic cultures is activity-dependent. Autapse (\blacktriangle) or heterosynapse (∇) densities are shown for every wild-type-mutant cell pair analyzed. When cultures are grown in the presence of (A) $1 \mu\text{M}$ TTX to block spontaneous action potentials [$n = 17$ cell pairs, total of 2,322 synapses (818 autapses and 1,504 heterosynapses)] or (B) $10 \mu\text{M}$ CNQX, $100 \mu\text{M}$ APV, and $50 \mu\text{M}$ PCTX to block postsynaptic receptors [$n = 10$ cell pairs, total of 2,699 synapses (929 autapses and 1,770 heterosynapses)], autapse and heterosynapse densities are scattered across the line with a slope of 1 (dotted line), and no noticeable bias is observed toward either wild-type or syp-mutant synapses.

Effect of Activity on Relative Synapse Formation Between Wild-Type and syp-Mutant Cells. The deficit in the extent of mutant synapse formation is apparent only in the presence of competing wild-type inputs. Because activity plays a role in synapse maturation (3), we next tested whether a chronic blockade of action potentials would reverse the observed reduction in syp-mutant synapses relative to wild-type synapses. Interestingly, cell pairs that were cultured in the continued presence of $1 \mu\text{M}$ TTX for 11–14 days no longer displayed the bias toward wild-type synapses over mutant synapses for symmetrical autapses and heterosynapses (Fig. 5A). Mean synapse densities per μm of dendrite for wild-type and mutant synapses were 0.15 ± 0.02 and 0.17 ± 0.02 , respectively, for autapses ($n = 17$, $P > 0.5$), and 0.18 ± 0.03 and 0.18 ± 0.02 , respectively, for heterosynapses ($n = 17$, $P > 0.9$). We also examined the effect of inhibiting postsynaptic receptors by culturing cells for 11–14 days in the presence of glutamate receptor blockers, CNQX ($10 \mu\text{M}$) and \pm APV ($100 \mu\text{M}$), and the GABA_A receptor blocker, PCTX ($50 \mu\text{M}$). Preventing postsynaptic receptor activation also reversed the observed bias in symmetrical autapses or heterosynapses formed between wild-type and mutant cells (Fig. 5B). Mean autapse densities per μm of dendrite for wild-type and mutant synapses were 0.20 ± 0.04 and 0.15 ± 0.04 , respectively ($n = 10$, $P > 0.3$), and mean heterosynapse densities were 0.25 ± 0.05 and 0.24 ± 0.05 , respectively ($n = 10$, $P > 0.8$). Neuronal activity, therefore, is required for the preferential formation of wild-type synapses over mutant synapses in heterogenotypic cell pairs.

Discussion

We present a simple method for studying competitive synapse formation in a heterogenotypic circuit formed between a wild-

type neuron and a neuron lacking syp in culture. Analysis of the heterogenotypic cell pairs revealed a significant activity-dependent reduction in the density of mutant synapses relative to wild-type synapses. Nevertheless, consistent with a previous study demonstrating that syp knockout mice have no detectable defects in brain anatomy and synaptic properties (9), we also did not find any impairment in the extent of synapse formation in homogenotypic syp-mutant cultures. The reduction in syp-mutant synapse density was more apparent for autapses than for heterosynapses. Because spontaneous activity plays a role in the reduction of mutant synapses, the timing of autapse activation may provide a more efficacious signal for reducing the rate of synapse formation or promoting synapse loss under competitive conditions (see below). Although the density of syp-mutant synapses was reduced, the combined density of autapses and heterosynapses formed onto either wild-type or mutant cells was comparable between the two cells in a heterogenotypic cell pair. Such conservation of the extent of synapse formation is reminiscent of homeostatic mechanisms that maintain the stability of neural networks (15). In this study, we did not distinguish between excitatory and inhibitory cells, as syp is expressed at both types of synapses. Nevertheless, the contribution of syp to synapse competition may differ among excitatory-excitatory, excitatory-inhibitory, and inhibitory-inhibitory cell pairs. Thus, it remains to be tested whether the lack of syp exerts differential reduction of mutant synapses, depending on the cell type involved.

Despite the recent progress in our understanding of the mechanisms of activity-dependent development of neuronal morphology and circuitry, the molecular mechanisms of central synapse formation still remain poorly understood. Although synaptic proteins are expected to participate in synaptogenesis and synapse remodeling, those with nonessential function(s) are difficult to identify. The competitive synapse formation assay based on the reciprocal two-cell circuit is beneficial for identifying a subtle role for synaptic proteins in directing synapse-circuit formation like that we demonstrate here for syp. The internal symmetry of autaptic and heterosynaptic connections in a two-cell closed circuit permits a direct comparison of the extent of synapse formation between a wild-type and a mutant cell. Furthermore, the synapse-specific mutation allows one to identify the mutant autapses and heterosynapses by differential immunofluorescence staining for a synaptic protein common to both genotypes and the protein missing in the mutant. Our assay is laborious in that it involves the identification of a wild-type-mutant cell pair based on DiI/DiO staining, and the reidentification of the same cell pair after processing for immunocytochemistry, to analyze synapse density. A similar assay based on

the synapse-specific proteins fused to green fluorescent protein (GFP) and its spectral variants will circumvent such problems.

How does syp regulate the extent of synapse formation? Syp is expressed at high levels during synaptogenesis and is one of the earliest synaptic proteins to accumulate at developing synapses in culture (16). Such properties of syp expression are compatible with its role in regulating synapse formation as found in this study. Because our analysis was limited to 11- to 14-day-old cultures, it remains possible that syp plays a role in synapse stabilization rather than synapse formation *per se*. Indeed, the extent of synapse formation was not impaired in syp-mutant homogenotypic cultures, indicating that the synaptogenesis machinery is still intact in the absence of syp. The observed reduction in the syp-mutant synapses in the presence of competing wild-type inputs thus may reflect impairment in the activity-dependent stabilization of newly formed synapses lacking syp. What are the potential mechanisms that preferentially favor the formation and/or maintenance of syp-positive wild-type synapses over the mutant synapses? Syp is phosphorylated by both Ser/Thr and Tyr kinases (17–19), and it is one of the major phosphotyrosine-containing proteins in the mature nerve terminal (20). Thus, it is possible that syp is a downstream target of a second messenger system which, when phosphorylated, promotes synapse stabilization by acting on other synaptic proteins required for maintaining the synaptic architecture. The upstream signal for initiating syp-dependent synapse stabilization may be neurotrophins, which have been implicated in various forms of activity-dependent regulation of synaptic connectivity (15, 21, 22).

Finally, although our results are consistent with the lack of phenotype in syp-mutant mice, they also indicate that animals may develop abnormalities if syp is expressed at different levels among neurons within a specific brain region. Interestingly, patients with schizophrenia show reduced levels of syp as opposed to control individuals (23). This fact suggests an interesting possibility, that a reduction in syp levels in a subset of neurons alters the neuronal circuitry, thereby contributing to the disease ontogeny. If such is the case, it would be important to identify the mechanisms that regulate the levels of expression of syp.

We thank Tom Südhof for kindly providing syp knockout-mice breeders and antibodies to syp, Steve Potter for advice on DiI/DiO staining, Miguel Morales for his generous help, Gilbert Ramirez for technical assistance, and Chuck Stevens, Tom Südhof, and the Goda lab members for comments on the manuscript. This work was supported by funds from the National Institutes of Health, the Whitehall Foundation, Damon Runyon-Walter Winchell Foundation, and The Alfred P. Sloan Foundation.

- Katz, L. C. & Callaway, E. M. (1992) *Annu. Rev. Neurosci.* **15**, 31–56.
- Goodman, C. S. & Shatz, C. J. (1993) *Cell* **72**, 77–98.
- Constantine-Paton, M. & Cline, H. T. (1998) *Curr. Opin. Neurobiol.* **8**, 139–148.
- Sanes, J. R. & Lichtman, J. W. (1999) *Annu. Rev. Neurosci.* **22**, 389–442.
- Marqueze-Pouey, B., Wisden, W., Malosio, M. L. & Betz, H. (1991) *J. Neurosci.* **11**, 3388–3397.
- Fykse, E. M., Takei, K., Walch-Solimena, C., Geppert, M., Jahn, R., De Camilli, P. & Südhof, T. C. (1993) *J. Neurosci.* **13**, 4997–5007.
- Alder, J., Xie, Z. P., Valtorta, F., Greengard, P. & Poo, M. (1992) *Neuron* **9**, 759–768.
- Daly, C., Sugimori, M., Moreira, J. E., Ziff, E. B. & Llinas, R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6120–6125.
- McMahon, H. T., Bolshakov, V. Y., Janz, R., Hammer, R. E., Siegelbaum, S. A. & Südhof, T. C. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4760–4764.
- Morales, M., Colicos, M. A. & Goda, Y. (2000) *Neuron* **27**, 539–550.
- Potter, S. M., Pine, J. & Fraser, S. E. (1996) *Scanning Microsc. Suppl.* **10**, 189–199.
- Segal, M. M. & Furshpan, E. J. (1990) *J. Neurophysiol.* **64**, 1390–1399.
- Bekkers, J. M. & Stevens, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7834–7838.
- Goda, Y. & Stevens, C. F. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 1283–1288.
- Turrigiano, G. G. (1999) *Trends Neurosci.* **22**, 221–227.
- Fletcher, T. L., Cameron, P., De Camilli, P. & Banker, G. (1991) *J. Neurosci.* **11**, 1617–1626.
- Barnekow, A., Jahn, R. & Schartl, M. (1990) *Oncogene* **5**, 1019–1024.
- Rubenstein, J. L., Greengard, P. & Czernik, A. J. (1993) *Synapse* **13**, 161–172.
- Linstedt, A. D., Vetter, M. L., Bishop, J. M. & Kelly, R. B. (1992) *J. Cell Biol.* **117**, 1077–1084.
- Pang, D. T., Wang, J. K., Valtorta, F., Benfenati, F. & Greengard, P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 762–766.
- Bonhoeffer, T. (1996) *Curr. Opin. Neurobiol.* **6**, 119–126.
- McAllister, A. K., Katz, L. C. & Lo, D. C. (1999) *Annu. Rev. Neurosci.* **22**, 295–318.
- Vawter, M. P., Howard, A. L., Hyde, T. M., Kleinman, J. E. & Freed, W. J. (1999) *Mol. Psychiatry* **4**, 467–475.